(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 November 2001 (15.11.2001)

PCT

(10) International Publication Number
WO 01/84937 A1

(51) International Patent Classification[7]: A01N 65/00, 63/02, 63/00 // (A01N 65/00, 43:84, 43:78, 43:42, 43:38, 37:40, 37:38, 35:10, 35:04, 33:26, 33:10, 31:16, 31:08) (A01N 33/06, 63:02, 43:84, 43:78, 43:42, 43:38, 37:40, 37:38, 35:10) (A01N 35/04, 33:26, 33:10, 33:06, 31:16, 31:08)

(21) International Application Number: PCT/DK01/00315

(22) International Filing Date: 7 May 2001 (07.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2000 00755 8 May 2000 (08.05.2000) DK

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/84937 A1

(54) Title: OXIDOREDUCTASE MEDIATED ANTIMICROBIAL ACTIVITY

(57) Abstract: The present invention relates to an enzymatic method for killing or inhibiting microbial cells or micro-organisms, e.g. in laundry, on hard surfaces, in water systems, on skin, on teeth or on mucous membranes. The present invention also relates to the use of said enzymatic composition for preserving food products, cosmetics, paints, coatings, etc.

OXIDOREDUCTASE MEDIATED ANTIMICROBIAL ACTIVITY

FIELD OF THE INVENTION

The present invention relates to an enzymatic antimicrobial method for killing or inhibiting microbial cells or micro-organisms present, e.g., in laundry, on hard surfaces, in water systems, on skin, on teeth or on mucous membranes. The present invention also relates to the use of an enzymatic antimicrobial composition for preserving food products, cosmetics, paints, coatings, etc.

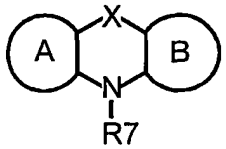
BACKGROUND

Various enzymatic antimicrobial compositions are known in the art. For instance, WO94/04127 discloses stabilized dentifrice compositions which are capable of producing antimicrobially effective concentrations of hypothiocyanite ions. The compositions contain an oxidoreductase capable of producing hydrogen peroxide and a peroxidase enzyme capable of oxidizing thiocyanate ions normally present in saliva to antimicrobial hypothiocyanite ions. Suitable peroxidases include lactoperoxidase, myeloperoxidase, salivary peroxidase and chloroperoxidase.

The object of the present invention is to provide an enhanced method for killing or inhibiting microbial cells.

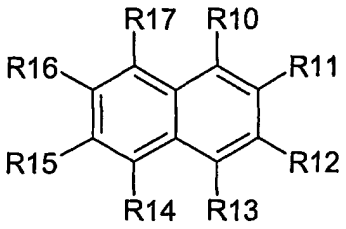
SUMMARY OF THE INVENTION

According to the present invention there is provided a method for killing or inhibiting microbial cells comprising treating said microbial cells with a phenol oxidizing enzyme system and an enhancing agent selected from the group consisting of:



C - X - **D**;

E - R6; and



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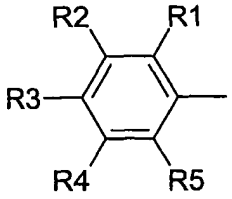
in which C, D, and E independently of each other are:



and R1, R2, R3, R4, R5, R6, R7, R10, R11, R12, R13, R14, R15, R16, R17 independently of each other are H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl; and X is a single bond or NH, NCH_3, NC_2H_5, O, S, N=N, CH=N, or CH=CH.

A and B are 6 membered aromatic rings, and may independently of each other be substituted with H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

One or more carbon atoms of the aromatic rings of A, B, C, D, and E may independently of each other be substituted with N or S, thus rendering said aromatic ring a heterocyclic ring.

In further aspects, the present invention relates to methods for killing or inhibiting microbial cells in laundry, in cosmetic products or on hard surfaces.

In still further aspects, the present invention relates to use of an enzymatic antimicrobial composition for cleaning of contact lenses, for cleaning of water systems, for preserving of paint, and in a cleaning-in-place system.

DETAILED DESCRIPTION

In the context of the present invention the term "antimicrobial" is intended to mean that there is a bactericidal and/or a bacteriostatic and/or fungicidal and/or fungistatic effect and/or a virucidal effect and/or a sporicidal effect, wherein

The term "bactericidal" is to be understood as capable of killing bacterial cells.

Bactericidal activity is measured as a logarithmic reduction (log reduction) in the number of living cells or Colony Forming Units pr. ml (CFU/ml), e.g. 1 log reduction corresponds to a reduction in the number of living cells of *Pseudomonas putida* ATCC12633 from Y x 10^X CFU/M (CFU: Colony Forming Units; M: ml or g) to Y x 10^{X-1} CFU/M, where X can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11, and Y can be any number from 0 to 10. The number of living cells are to be determined as the number of *Pseudomonas putida* ATCC12633, which can grow on LB Agar (#0285, Merck, Germany) plates at 30°C.

The term "bacteriostatic" is to be understood as capable of inhibiting bacterial growth, i.e. inhibiting growing bacterial cells.

The term "fungicidal" is to be understood as capable of killing fungal cells.

The term "fungistatic" is to be understood as capable of inhibiting fungal growth, i.e. inhibiting growing fungal cells.

The term "virucidal" is to be understood as capable of inactivating virus.

The term "sporicidal" is to be understood as capable of inactivating spores.

The term "microbial cells" denotes bacterial or fungal cells, and the term "micro-organism" denotes a fungus (including yeasts) or a bacterium.

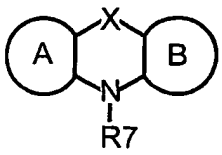
In the context of the present invention the term "inhibiting growth of microbial cells" is intended to mean that the cells are in the non-growing state, i.e., that they are not able to propagate.

The "phenol oxidizing enzyme system" describes an enzyme possessing peroxidase activity together with a hydrogen peroxide source, or a laccase or laccase related enzyme together with oxygen.

The term "hard surface" as used herein relates to any surface, which is essentially non-permeable for micro-organisms. Examples of hard surfaces are surfaces made from metal, e.g., stainless steel, plastics, rubber, board, glass, wood, paper, textile, concrete, rock, marble, gypsum and ceramic materials which optionally may be coated, e.g., with paint, enamel and the like. The hard surface can also be a process equipment, e.g., a cooling tower, an osmotic membrane, a water treatment plant, a dairy, a food processing plant, a chemical or pharmaceutical process plant. Accordingly, the composition according to the present invention is useful in a conventional cleaning-in-place (C-I-P) system.

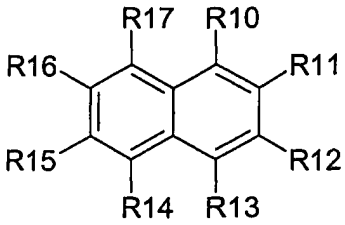
Enhancing agent

The present invention relates to enhancing agents selected from the group consisting of:



C - X - **D**;

E - R6; and



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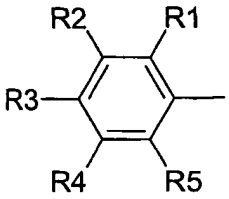
in which C, D, and E independently of each other are:



and R1, R2, R3, R4, R5, R6, R7, R10, R11, R12, R13, R14, R15, R16, R17 independently of each other are H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl; and X is a single bond or NH, NCH_3, NC_2H_5, O, S, N=N, CH=N, or CH=CH. In a preferred embodiment R1, R2, R3, R4, R5, R6, R7, R10, R11, R12, R13, R14, R15, R16, R17 independently of each other are H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl; and X is a single bond or NH, NCH_3, NC_2H_5, O, S, N=N, CH=N, or CH=CH.

A and B are six membered aromatic rings, and may independently of each other be substituted with H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl. In a preferred embodiment A and B may independently of each other be substituted with H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

One or more carbon atoms of the aromatic rings of A, B, C, D, E, and F may independently of each other be substituted with N or S, thus rendering said aromatic ring a heterocyclic ring.

In an embodiment X is a single bond or NH, NCH_3, NC_2H_5, O, S, N=N, CH=N, or CH=CH; preferably X is a single bond or NH, NCH_3, NC_2H_5, O, N=N, CH=N, or CH=CH; preferably X is a single bond or NH, NCH_3, NC_2H_5, S, N=N, CH=N, or CH=CH; more preferably X is a single bond or NH, NCH_3, NC_2H_5, N=N, CH=N, or CH=CH.

In an embodiment the substituents of A are independently of each other H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl. Preferably the substituents of A are independently of each other H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

In an embodiment the substituents of B are independently of each other H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl. Preferably the substituents of B are independently of each other H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

In an embodiment R1, R2, R3, R4, and R5 of C are independently of each other H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl. Preferably R1, R2, R3, R4, and R5 of C are independently of each other H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

In an embodiment R1, R2, R3, R4, and R5 of D are independently of each other H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl. Preferably R1, R2, R3, R4, and R5 of D are independently of each other H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

In an embodiment R1, R2, R3, R4, and R5 of E are independently of each other

H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl. Preferably R1, R2, R3, R4, and R5 of E are independently of each other H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

In an embodiment R10, R11, R12, R13, R14, R15, R16 and R17 of F are independently of each other H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl. Preferably R10, R11, R12, R13, R14, R15, R16 and R17 of F are independently of each other H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

The terms "C_{1-n}-alkyl" wherein n can be from 1 through 8, as used herein, represent a branched or straight, saturated or unsaturated alkyl group having from one to the specified number of carbon atoms. Typical C_{1-6}-alkyl groups include, but are not limited to, methyl, ethyl, ethenyl (vinyl), *n*-propyl, iso-propyl, propenyl, isopropenyl, butyl, iso-butyl, *sec*-butyl, *tert*-butyl, crotyl, methallyl, pentyl, iso-pentyl, prenyl, hexyl, iso-hexyl and the like.

The term "acyl" as used herein refers to a monovalent substituent comprising a C_{1-6}-alkyl group linked through a carbonyl group; such as e.g. acetyl, propionyl, butyryl, isobutyryl, pivaloyl, valeryl, and the like.

In an embodiment at least one of the substituents of A are H, preferably at least two of the substituents of A are H, more preferably at least three of the substituents of A are H, most preferably at least four of the substituents of A are H, in particular all the substituents of A are H.

In another embodiment at least one of the substituents of B are H, preferably at least two of the substituents of B are H, more preferably at least three of the substituents of B are H, most preferably at least four of the substituents of B are H, in particular all the substituents of B are H.

In another embodiment at least one of the substituents R1, R2, R3, R4, and R5 of C are H, preferably at least two of the substituents R1, R2, R3, R4, and R5 of C are H, more preferably at least three of the substituents R1, R2, R3, R4, and R5 of C are H, most preferably at least four of the substituents R1, R2, R3, R4, and R5 of C are H, in particular all the substituents R1, R2, R3, R4, and R5 of C are H.

In another embodiment at least one of the substituents R1, R2, R3, R4, and R5 of D are H, preferably at least two of the substituents R1, R2, R3, R4, and R5 of D are H, more preferably at least three of the substituents R1, R2, R3, R4, and R5 of D are H, most preferably at least four of the substituents R1, R2, R3, R4, and R5 of D are H, in particular all the substituents R1, R2, R3, R4, and R5 of D are H.

In another embodiment at least one of the substituents R1, R2, R3, R4, and R5 of E are H, preferably at least two of the substituents R1, R2, R3, R4, and R5 of E are H, more preferably at least three of the substituents R1, R2, R3, R4, and R5 of E are H, most preferably at least four of the substituents R1, R2, R3, R4, and R5 of E are H, in particular all the substituents R1, R2, R3, R4, and R5 of E are H.

In another embodiment at least one of the substituents R10, R11, R12, R13, R14, R15, R16, and R17 of F are H, preferably at least two of the substituents R10, R11, R12, R13, R14, R15, R16, and R17 of F are H, more preferably at least three of the substituents R10, R11, R12, R13, R14, R15, R16, and R17 of F are H, more preferably at least four of the substituents R10, R11, R12, R13, R14, R15, R16, and R17 of F are H, more preferably at least five of the substituents R10, R11, R12, R13, R14, R15, R16, and R17 of F are H, more preferably at least seven of the substituents R10, R11, R12, R13, R14, R15, R16, and R17 of F are H, most preferably at least six of the substituents R10, R11, R12, R13, R14, R15, R16, and R17 of F are H, in particular all the substituents R10, R11, R12, R13, R14, R15, R16, and R17 of F are H.

In particular embodiments according to the invention the enhancing agent is selected from the group consisting of:

4-aminophenol;

p-Coumaric acid;

4,4'-Biphenol;

3,3',5,5'-Tetramethylbenzidine;

4,4'-Diaminodiphenylamine sulfate;

4,4'-Dimethoxy-N-methyl-diphenylamine;

4,4'-Dihydroxydiphenyl ether;

4-Hydroxy-4'-dimethylamino azobenzene;

N'-Benzylidene-N,N-dimethyl-p-phenylenediamine;

4-Amino-4'-hydroxystilbene;

4'-Bromo-4-(dimethylamino)-chalcone;
3-Dimethylamino-9-ethylcarbazole;
Harmine, HCl H_2O;
Methylsyringate;
Propyl sinapate;
1,5-Diaminonaphthalene;
1,5-Dihydroxynaphthalene;
2,7-Dihydroxynaphthalene;
7-Methoxy-2-naphthol;
2-Hydroxy-1-naphthaldehyde;
2-Hydroxy-1-naphthoic acid;
8-Aminoquinoline;
10-Methylphenoxazine; and
3,7-Dibromophenoxazine-10-propionic acid.

The enhancing agent of the invention may be present in concentrations of from 1 to 1000 μM, preferably of from 5 to 500 μM, and more preferably from 10 to 200 μM.

Hydrogen peroxide/Oxygen

If the phenol oxidizing enzyme requires a source of hydrogen peroxide, the source may be hydrogen peroxide or a hydrogen peroxide precursor for in situ production of hydrogen peroxide, e.g., percarbonate or perborate, or a hydrogen peroxide generating enzyme system, (e.g., an oxidase together with a substrate for the oxidase, e.g., an amino acid oxidase together with a suitable amino acid), or a peroxycarboxylic acid or a salt thereof. Hydrogen peroxide may be added at the beginning of or during the process, e.g., typically in an amount corresponding to levels of from 0.001-25 mM, preferably to levels of from 0.005-5 mM, and particularly to levels of from 0.01-1 mM.

If the phenol oxidizing enzyme requires molecular oxygen, molecular oxygen from the atmosphere will usually be present in sufficient quantity. If more O_2 is needed, additional oxygen may be added.

Phenol Oxidizing Enzyme

In the context of the present invention the enzyme of the phenol oxidizing enzyme may be an enzyme possessing peroxidase activity or a laccase or a laccase related enzyme.

The enzyme of the invention may typically be present in concentrations of from 1 to 100000 μg enzyme protein per liter aqueous solution, preferably of from 5 to 50000 μg enzyme protein per liter aqueous solution, more preferably of from 10 to 10000 μg enzyme protein per liter aqueous solution, and most preferably of from 50 to 5000 μg enzyme protein per liter aqueous solution.

Peroxidases and Compounds possessing Peroxidase Activity

Compounds possessing peroxidase activity may be any peroxidase enzyme comprised by the enzyme classification (EC 1.11.1.7), or any fragment derived therefrom, exhibiting peroxidase activity.

Preferably, the peroxidase according to the invention is producible by plants (e.g. horseradish or soybean peroxidase) or micro-organisms such as fungi or bacteria.

Some preferred fungi include strains belonging to the subdivision *Deuteromycotina*, class *Hyphomycetes*, e.g., *Fusarium*, *Humicola*, *Tricoderma*, *Myrothecium*, *Verticillum*, *Arthromyces*, *Caldariomyces*, *Ulocladium*, *Embellisia*, *Cladosporium* or *Dreschlera*, in particular *Fusarium oxysporum* (DSM 2672), *Humicola insolens*, *Trichoderma resii*, *Myrothecium verrucaria* (IFO 6113), *Verticillum alboatrum*, *Verticillum dahlie*, *Arthromyces ramosus* (FERM P-7754), *Caldariomyces fumago*, *Ulocladium chartarum*, *Embellisia alli* or *Dreschlera halodes*.

Other preferred fungi include strains belonging to the subdivision *Basidiomycotina*, class *Basidiomycetes*, e.g., *Coprinus*, *Phanerochaete*, *Coriolus* or *Trametes*, in particular *Coprinus cinereus* f. *microsporus* (IFO 8371), *Coprinus macrorhizus*, *Phanerochaete chrysosporium* (e.g. NA-12) or *Trametes* (previously called *Polyporus*), e.g., *T. versicolor* (e.g. PR4 28-A).

Further preferred fungi include strains belonging to the subdivision *Zygomycotina*, class *Mycoraceae*, e.g., *Rhizopus* or *Mucor*, in particular *Mucor hiemalis*.

Some preferred bacteria include strains of the order *Actinomycetales*, e.g. *Streptomyces spheroides* (ATTC 23965), *Streptomyces thermoviolaceus* (IFO 12382)

or *Streptoverticillum verticillium* ssp. *verticillium*.

Other preferred bacteria include *Rhodobacter sphaeroides*, *Rhodomonas palustri*, *Streptococcus lactis*, *Pseudomonas purrocinia* (ATCC 15958), *Pseudomonas fluorescens* (NRRL B-11) and *Bacillus* strains, e.g. *Bacillus pumilus* (ATCC 12905) and *Bacillus stearothermophilus*.

Further preferred bacteria include strains belonging to *Myxococcus*, e.g., *M. virescens*.

The peroxidase may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said peroxidase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the peroxidase, in a culture medium under conditions permitting the expression of the peroxidase and recovering the peroxidase from the culture.

Particularly, a recombinantly produced peroxidase is a peroxidase derived from a *Coprinus* sp., in particular *C. macrorhizus* or *C. cinereus* according to WO 92/16634.

In the context of this invention, compounds possessing peroxidase activity comprise peroxidase enzymes and peroxidase active fragments derived from cytochromes, haemoglobin or peroxidase enzymes.

Determination of Peroxidase Activity (POXU)

One peroxidase unit (POXU) is the amount of enzyme which under the following conditions catalyze the conversion of 1 µmole hydrogen peroxide per minute:

0.1 M phosphate buffer pH 7.0
0.88 mM hydrogen peroxide
1.67 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS)
30°C

The reaction is followed for 60 seconds (15 seconds after mixing) by the change in absorbance at 418 nm, which should be in the range 0.15 to 0.30.

For calculation of activity is used an absorption coefficient of oxidized ABTS of 36 mM^{-1} cm^{-1} and a stoichiometry of one µmole H_2O_2 converted per two µmole ABTS oxidized.

Laccases and Laccase Related Enzymes

In the context of this invention, laccases and laccase related enzymes comprise any laccase enzyme comprised by the enzyme classification (EC 1.10.3.2), any catechol oxidase enzyme comprised by the enzyme classification (EC 1.10.3.1), any bilirubin oxidase enzyme comprised by the enzyme classification (EC 1.3.3.5) or any monophenol monooxygenase enzyme comprised by the enzyme classification (EC 1.14.18.1).

The above-mentioned enzymes may be microbial, i.e. derived from bacteria or fungi (including filamentous fungi and yeasts), or they may be derived from plants.

Suitable examples from fungi include a laccase derivable from a strain of *Aspergillus*, *Neurospora*, e.g., *N. crassa*, *Podospora*, *Botrytis*, *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, e.g., *T. villosa* and *T. versicolor*, *Rhizoctonia*, e.g., *R. solani*, *Coprinus*, e.g., *C. cinereus*, *C. comatus*, *C. friesii*, and *C. plicatilis*, *Psathyrella*, e.g., *P. condelleana*, *Panaeolus*, e.g., *P. papilionaceus*, *Myceliophthora*, e.g., *M. thermophila*, *Schytalidium*, e.g., *S. thermophilum*, *Polyporus*, e.g., *P. pinsitus*, *Pycnoporus*, e.g. *P. cinnabarinus*, *Phlebia*, e.g., *P. radita* (WO 92/01046), or *Coriolus*, e.g., *C. hirsutus* (JP 2-238885).

Suitable examples from bacteria include a laccase derivable from a strain of *Bacillus*.

A laccase derived from *Coprinus*, *Myceliophthora*, *Polyporus*, *Pycnoporus*, *Scytalidium* or *Rhizoctonia* is preferred; in particular a laccase derived from *Coprinus cinereus*, *Myceliophthora thermophila*, *Polyporus pinsitus*, *Pycnoporus cinnabarinus*, *Scytalidium thermophilum* or *Rhizoctonia solani*.

The laccase or the laccase related enzyme may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said laccase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the laccase, in a culture medium under conditions permitting the expression of the laccase enzyme, and recovering the laccase from the culture.

Determination of Laccase Activity (LACU)

Laccase activity is determined from the oxidation of syringaldazin under aerobic

conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19 mM syringaldazin, 23 mM acetate buffer, pH 5.5, 30°C, 1 min. reaction time.

1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1.0 μmole syringaldazin per minute at these conditions.

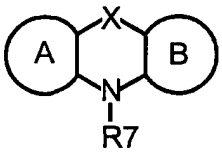
Determination of Laccase Activity (LAMU)

Laccase activity is determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19 mM syringaldazin, 23 mM Tris/maleate buffer, pH 7.5, 30°C, 1 min. reaction time.

1 laccase unit (LAMU) is the amount of enzyme that catalyses the conversion of 1.0 μmole syringaldazin per minute at these conditions.

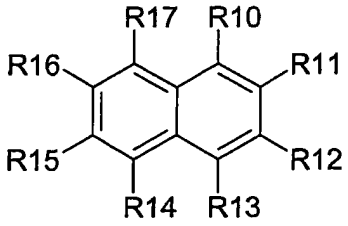
The composition

The present invention provides an enzymatic antimicrobial composition comprising a phenol oxidizing enzyme system and an enhancing agent of a formula selected from group consisting of:



C - X - **D**;

E - R6; and



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as described above.

The antimicrobial composition according to the invention may be formulated as a solid or a liquid.

When formulated as a liquid, the composition is typically an aqueous composition.

When formulated as a solid, the composition is typically a powder, a granulate, a paste or a gelled product.

It is preferred to use a two-part formulation system in the cases where hydrogen peroxide is needed, whereby the hydrogen peroxide is separate from the other components.

The composition of the invention may further comprise auxiliary agents such as wetting agents, thickening agents, buffer, stabilisers, perfume, colourants, fillers and the like.

Useful wetting agents are surfactants, i.e., non-ionic, anionic, amphoteric or zwitterionic surfactants.

The composition of the invention may be a concentrated product or a ready-to-use product. In use, the concentrated product is typically diluted with water to provide a medium having an effective antimicrobial activity, applied to the object to be disinfected or preserved, and allowed to react with the micro-organisms present.

The optimum pH of such an aqueous composition is usually a compromise between the optimum stability and optimum activity of the enzyme in question. In one aspect of the invention pH is in the range of pH 3 to 10.5 (such as pH 4 to 6 or pH 8 to 10), and in another aspect of the invention pH is in the range of pH 4 to 10, preferably pH 5 to 9, and more preferably pH 6 to 8.

In an embodiment, the present invention also provides a medical catheter comprising the composition of the invention.

The method

The present invention also provides a method for killing or inhibiting microbial cells comprising treating said microbial cells with the composition of the invention. Said treatment may be carried out with an effective amount of said composition.

As an "effective amount" is meant an amount suitable for obtaining the required antimicrobial effect in the chosen application; e.g. to reduce the number of living cells to 10%, 1% or less than 1%; or to prevent the number of living cells from doubling during 12 hours, 1 day, 5 days, 30 days or more than 30 days.

The composition of the invention may be capable of reducing the number of living cells (killing) of *E. coli* (DSM1576) to less than 50% (preferably less than 75%, more preferably less than 90%, most preferably less than 95%, in particular at least

99%), when incubated 10 min. at 20°C in an aqueous solution containing 1 mg/L of the composition.

The composition may also be capable of increasing the time before outgrowth (inhibition) of *E. coli* (DSM1576) at 25°C in a microbial growth substrate containing 1 mg/L of the composition by at least 5%, preferably at least 10%, more preferably at least 25%, most preferably at least 50%, and in particular at least 100%.

Uses

The composition of the invention may be incorporated into a detergent or cleaning composition typically comprising other enzyme types as well (see below).

The composition of the invention can also be used for inhibiting micro-organisms present in laundry, by treating the laundry with a soaking, washing or rinsing liquor comprising an effective amount of the composition.

When used for preservation of paint, food, beverages, cosmetics such as lotions (e.g. eye lotions), liquids, creams, gels, pastes, ointments (e.g. eye ointments), soaps, shampoos, conditioners, antiperspirants, deodorants, mouth wash, nasal sprays, contact lens products, enzyme formulations, or food ingredients, the composition used in the method of the present invention may be incorporated into e.g. water based paint, unpreserved food, beverages, cosmetics, contact lens products, food ingredients or anti-inflammatory product in an amount effective for killing or inhibiting growth of microbial cells.

In particular, the composition of the invention may be used as a preservation agent or a disinfection agent in water based paints (see below).

Furthermore, the composition according to the present invention may by useful as a disinfectant, e.g., in the treatment of acne, infections in the eye or the mouth, skin infections; in antiperspirants or deodorants; in foot bath salts; for cleaning and disinfection of contact lenses, hard surfaces, teeth (oral care), wounds, bruises and the like.

In general the composition of the present invention is useful for cleaning, disinfecting or inhibiting microbial growth on any hard surface. Examples of surfaces, which may advantageously be contacted with the composition of the invention are surfaces of process equipment used, e.g., in dairies, chemical or pharmaceutical process plants, water sanitation systems, paper pulp processing plants, water

treatment plants, and cooling towers. The composition of the invention may be used in an amount, which is effective for cleaning, disinfecting or inhibiting microbial growth on the surface in question.

In particular, the composition of the invention may be used for disinfecting and inhibiting microbial growth in paper and pulp processing plants.

Further, it is contemplated that the composition of the invention can advantageously be used in a cleaning-in-place (C.I.P.) system for cleaning of process equipment of any kind.

The method of the invention may additionally be used for cleaning surfaces and cooking utensils in food processing plants and in any area in which food is prepared or served such as hospitals, nursing homes, restaurants, especially fast food restaurants, delicatessens and the like. It may also be used as an antimicrobial in food products and would be especially useful as a surface antimicrobial for cheese, fruits and vegetables and for food in salad bars.

The composition of the present invention is also useful for microbial control of water lines, and for disinfection of water, in particular for disinfection of industrial water.

In an embodiment, the composition of the present invention is also useful for treating medical catheters to kill or inhibit microbes present on the surface of said medical catheters. The composition of the invention can also be used for treating wounds.

Conservation/preservation of paints

Conservation of paint products in cans has in the art been accomplished by adding non-enzymatic organic biocides to the paints. In the context of the invention paint is construed as a substance comprising a solid coloring matter dissolved or dispersed in a liquid vehicle such as water, organic solvent and/or oils, which when spread over a surface, dries to leave a thin colored, decorative and/or protective coating. Typically isothiazoliones, such as 5-chlor-2-methyl-4-thia-zoli-3-on, has been added to the paint as biocides to inhibit/prevent microbial growth in the paint. The method of the invention can however suitably be applied in this field, thereby solving the problem of the ever present environmental bio-hazards of using toxic organic biocides by replacing these toxic biocides with environmentally compatible enzymes. Thus the present invention provides a method for conservation of a paint comprising

contacting said paint with a phenol oxidizing enzyme and an enhancing agent according to the invention. Further the invention provides a paint composition comprising a phenol oxidizing enzyme and an enhancing agent as described in the present invention.

The paint is preferably a water based paint, *i.e.* the solids of the paint is dispersed in an aqueous solution. The paint may contain 0-20 % organic solvent, preferable 0-10%, e.g. 0-5%.

The enzyme may be added to the paint in an amount of 0.0001-100 mg active enzyme protein per litre paint, preferably 0.001-10 mg/l, e.g. 0.01-5 mg/l, while the enhancing agent may be added in an amount of 10-500 μM, preferably 25-250 μM, e.g. 100 μM of the paint composition.

Detergent Compositions

The antimicrobial composition of the invention may be added to and thus become a component of a detergent composition.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

In a specific aspect, the invention provides a detergent additive comprising the antimicrobial composition of the invention. The detergent additive as well as the detergent composition may comprise one or more other enzymes such as a protease, a lipase, a cutinase, an amylase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a laccase, and/or a peroxidase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are

included. The protease may be a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include Alcalase™, Savinase™, Primase™, Duralase™, Esperase™, and Kannase™ (Novozymes A/S), Maxatase™, Maxacal™, Maxapem™, Properase™, Purafect™, Purafect OxP™, FN2™, and FN3™ (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g. from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g. from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas sp.* strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g. from *B. subtilis* (Dartois et al. (1993), Biochemica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include Lipolase™ and Lipolase Ultra™ (Novozymes A/S).

Amylases: Suitable amylases (α and/or β) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, α-amylases obtained from *Bacillus*, e.g. a special strain of *B.*

licheniformis, described in more detail in GB 1,296,839.

Examples of useful amylases are the variants described in WO 94/02597, WO 94/18314, WO 96/23873, and WO 97/43424, especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™ and BAN™ (Novozymes A/S), Rapidase™ and Purastar™ (from Genencor International Inc.).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus, Pseudomonas, Humicola, Fusarium, Thielavia, Acremonium,* e.g. the fungal cellulases produced from *Humicola insolens, Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include Celluzyme™, and Carezyme™ (Novozymes A/S), Clazinase™, and Puradax HA™ (Genencor International Inc.), and KAC-500(B)™ (Kao Corporation).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g. from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separate additive or a combined additive, can be formulated e.g. as a granulate, a liquid, a

slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight.

When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The detergent may contain 0-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, carbonate, citrate,

nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinylpyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid co-polymers.

The detergent may contain a bleaching system, which may comprise a H_2O_2 source such as perborate or percarbonate, which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine or nonanoyloxybenzenesulfonate. Alternatively, the bleaching system may comprise peroxyacids of e.g. the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

The present invention is further illustrated in the following examples, which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLE 1

Antimicrobial activity of enhancing agents

Screening for enhancing agents with antimicrobial activity was performed in 384 well microtiter plates by measuring the ability of laccase / enhancing agent combinations to inhibit growth of *Pseudomonas putida* ATCC12633.

Enhancing agents were dissolved in EtOH at a concentration of 10 mM and stored at 4°C. From these stock solutions a 96 well master plate containing ~200 µM enhancing agent was generated by adding 6 µl of enhancing agent stock into 280 µl milliQ water. The layout of the master plate was as shown in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hepes	Hepes	m1	m1	m9	M9	m17	m17	m25	m25	m33	m33
B	Hepes	Hepes	m2	m2	m10	M10	m18	m18	m26	m26	m34	m34
C	Hepes	Hepes	m3	m3	m11	M11	m19	m19	m27	m27	m35	m35
D	Hepes	Hepes	m4	m4	m12	M12	m20	m20	m28	m28	m36	m36
E	Hepes	Hepes	m5	m5	m13	M13	m21	m21	m29	m29	m37	m37
F	Hepes	Hepes	m6	m6	m14	M14	m22	m22	m30	m30	m38	m38
G	Hepes	Hepes	m7	m7	m15	M15	m23	m23	m31	m31	m39	m39
H	Hepes	Hepes	m8	m8	m16	M16	m24	m24	m32	m32	m40	m40

Table 1. Layout of master plate containing enhancing agent. Columns 1 and 2 contain 50mM HEPES buffer pH 7.0 and m1-m40 are numbered enhancing agents.

Pseudomonas putida ATCC12633 was grown in a dilution series over night in LB medium and a late exponential culture was harvested by centrifugation (5 min @ 4000 RPM in a Microcentrifuge 154, Ole Dich, Denmark) and washed twice in 50 mM HEPES buffer pH 7.0. OD@490 was adjusted to 0.05 corresponding to approximately 10E5 cells/ml.

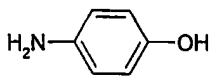
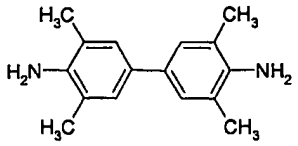
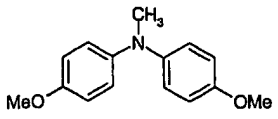
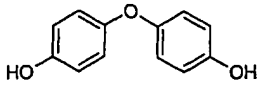
	1	2	3	4	5	6	7	8	9	10	11	12
A	Hepes	Hepes	m1	m1	m9	m9	m17	m17	m25	m25	m33	m33
B	Hepes	Hepes	m1	m1	m9	m9	m17	m17	m25	m25	m33	m33
C	10E0	10E0	m2	m2	m10	m10	m18	m18	m26	m26	m34	m34
D	10E0	10E0	m2	m2	m10	m10	m18	m18	m26	m26	m34	m34
E	10E1	10E1	m3	m3	m11	m11	m19	m19	m27	m27	m35	m35
F	10E1	10E1	m3	m3	m11	m11	m19	m19	m27	m27	m35	m35
G	10E2	10E2	m4	m4	m12	m12	m20	m20	m28	m28	m36	m36
H	10E2	10E2	m4	m4	m12	m12	m20	m20	m28	m28	m36	m36
I	10E3	10E3	m5	m5	m13	m13	m21	m21	m29	m29	m37	m37
J	10E3	10E3	m5	m5	m13	m13	m21	m21	m29	m29	m37	m37
K	Hepes	Hepes	m6	m6	m14	m14	m22	m22	m30	m30	m38	m38
L	Hepes	Hepes	m6	m6	m14	m14	m22	m22	m30	m30	m38	m38
M	LB	LB	m7	m7	m15	m15	m23	m23	m31	m31	m39	m39
N	LB	LB	m7	m7	m15	m15	m23	m23	m31	m31	m39	m39
O			m8	m8	m16	m16	m24	m24	m32	m32	m40	m40
P			m8	m8	m16	m16	m24	m24	m32	m32	m40	m40

Table 2. Layout of screenings plate. Only half of the 384 well plate is shown. Rows 3, 5, 7, 9, 11 contain enhancing agent only, whereas rows 4, 6, 8, 10, 12 contain enhancing agent and laccase. Rows 1 and 2 are negative controls as well as a

dilution series of cells are included in order to evaluate the amount of killing observed by the laccase / enhancing agent system. All assays were run in duplicate.

10 µl of the enhancing agent from the 96 well master plate (Table 1) was transferred to a 384 well plate in duplicate as shown in table 2. The 384 well screening plate was pre-incubated at 40°C on a heat block (Techne DB-2A, Buch & Holm A/S, Denmark) before the assay was started by addition of 35 µl of preheated buffer (40°C) containing cells (10E5 CFU/ml) with or without laccase (12,5 µg/ml). The reaction was incubated for 25 min at 40°C, and 45 µl LB medium was added to allow cells to grow. Growth at 30°C was monitored using a Polarstar Galaxy microtitter plate reader (BMG Labtechnologies, Germany) in the absorbance mode. The development in absorbance was measured online for 16 hours at 480 nm, and the resulting growth curves were used for evaluation of the anti-microbial activity of the different laccase / enhancing agent combinations. The reduction in colony forming units was estimated by comparison to the standard curve, and anti-microbial enhancing agents were categorized in two groups: Group 1 compounds showed between 10E2 and 10E5 reduction and group 2 showed more than 10E5 reduction (i.e. total kill). Results are shown in tables 3-6.

Table 3. Enhancing agents with antimicrobial activity mediated by MtL:

CAS no.	Name		Supplier	CFU reduction
123-30-8	4-aminophenol		Aldrich	> 10^5
54827-17-7	3,3',5,5'-Tetra-methylbenzidine		Acros	10^2-10^5
27151-57-1	4,4'-Dimethoxy-N-methyldiphenyl-amine		Lancaster	10^2-10^5
1965-09-9	4,4'-Dihydroxy-diphenyl ether		Sigma-Aldrich (Acros)	> 10^5

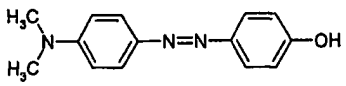
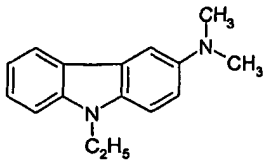
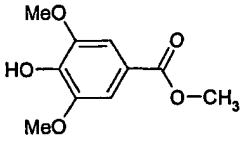
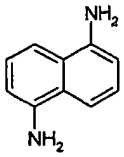
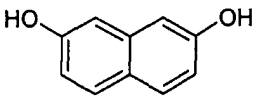
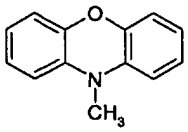
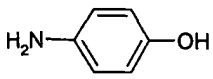
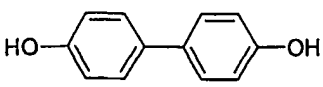
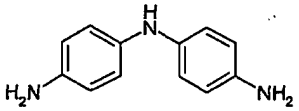
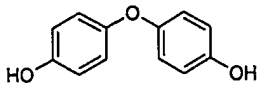
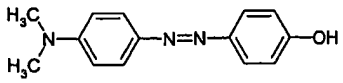
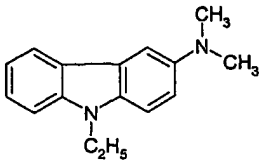
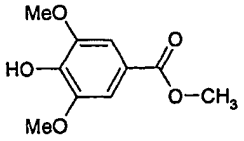
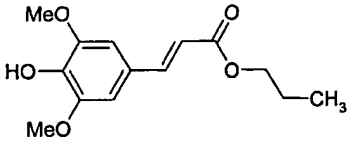
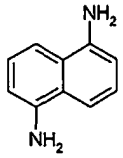
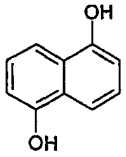
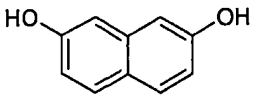
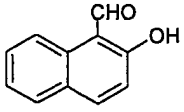
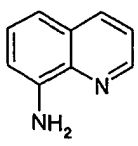
2496-15-3	4-Hydroxy-4'-dimethylamino azobenzene		TCI	> 10^5
	3-Dimethylamino-9-ethylcarbazole		Novozymes	> 10^5
884-35-5	Methylsyringate		Lancaster	10^2-10^5
2243-62-1	1,5-Diamino-naphthalene		Novozymes	> 10^5
582-17-2	2,7-Dihydroxy-naphthalene		Novozymes	10^2-10^5
25782-99-4	10-Methyl-phenoxazine		Novozymes	10^2-10^5

Table 4. Enhancing agents with antimicrobial activity mediated by RsL:

CAS no.	Name		Supplier	CFU reduction
123-30-8	4-aminophenol		Aldrich	> 10^5
92-88-6	4,4'-Biphenol, 97%		Aldrich	10^2-10^5
6369-04-6	4,4'-Diamino-diphenylamine sulfate, tech. 85%		Janssen Chemica	> 10^5

1965-09-9	4,4'-Dihydroxy-diphenyl ether		Sigma-Aldrich (Acros)	> 10^5
2496-15-3	4-Hydroxy-4'-dimethylamino azobenzene		TCI	> 10^5
	3-Dimethylamino-9-ethylcarbazole		Novozymes	> 10^5
884-35-5	Methylsyringate		Lancaster	10^2-10^5
	Propyl sinapate		Novozymes	> 10^5
2243-62-1	1,5-Diamino-naphthalene		Novozymes	> 10^5
83-56-7	1,5-Dihydroxy-naphthalene		Novozymes	10^2-10^5
582-17-2	2,7-Dihydroxy-naphthalene		Novozymes	10^2-10^5
2283-08-1	2-Hydroxy-1-naphthoic acid		TCI	> 10^5
578-66-5	8-Aminoquinoline		Aldrich	> 10^5

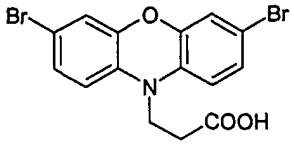
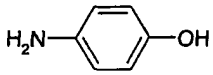
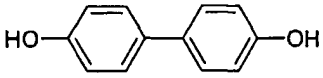
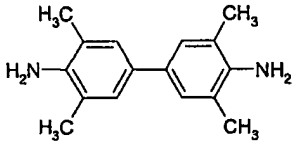
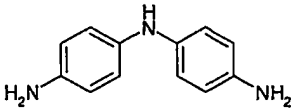
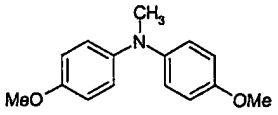
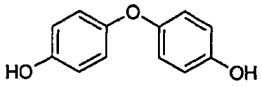
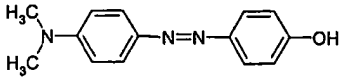
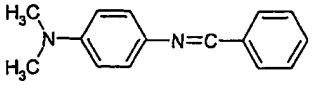
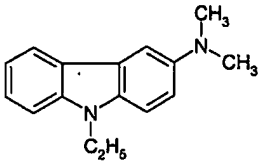
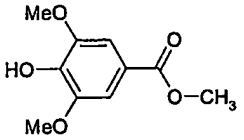
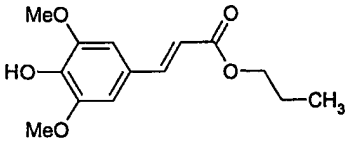
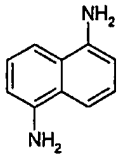
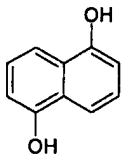
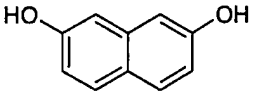
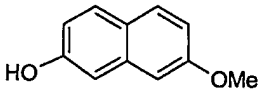
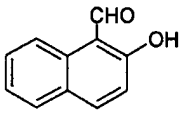
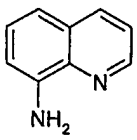
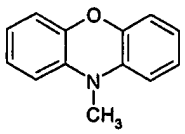
	3,7-Dibromo-phenoxazine-10-propionic acid		Novozymes	> 10^5

Table 5. Enhancing agents with anti-microbial activity mediated by CcL:

CAS no.	Name		Supplier	CFU reduction
123-30-8	4-aminophenol		Aldrich	> 10^5
92-88-6	4,4'-Biphenol, 97%		Aldrich	10^2-10^5
54827-17-7	3,3',5,5'-Tetra-methylbenzidine		Acros	10^2-10^5
6369-04-6	4,4'-Diamino-diphenylamine sulfate, tech. 85%		Janssen Chemica	> 10^5
27151-57-1	4,4'-Dimethoxy-N-methyldiphenyl-amine		Lancaster	10^2-10^5
1965-09-9	4,4'-Dihydroxy-diphenyl ether		Sigma-Aldrich (Acros)	> 10^5
2496-15-3	4-Hydroxy-4'-dimethylamino azobenzene		TCI	> 10^5
889-37-2	N'-Benzylidene-N,N-dimethyl-p-phenylenediamine		Sigma-Aldrich	10^2-10^5

	3-Dimethylamino-9-ethylcarbazole		Novozymes	> 10^5
884-35-5	Methylsyringate		Lancaster	10^2-10^5
	Propyl sinapate		Novozymes	> 10^5
2243-62-1	1,5-Diamino-naphthalene		Novozymes	> 10^5
83-56-7	1,5-Dihydroxy-naphthalene		Novozymes	10^2-10^5
582-17-2	2,7-Dihydroxy-naphthalene		Novozymes	10^2-10^5
5060-82-2	7-Methoxy-2-naphthol, 98%		Aldrich	10^2-10^5
2283-08-1	2-Hydroxy-1-naphthoic acid		TCI	> 10^5
578-66-5	8-Aminoquinoline		Aldrich	> 10^5
25782-99-4	10-Methyl-phenoxazine		Novozymes	10^2-10^5

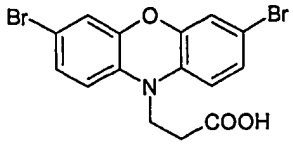
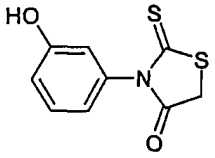
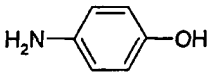
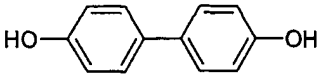
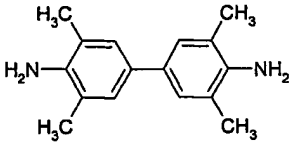
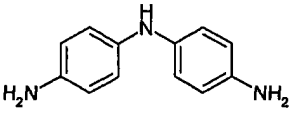
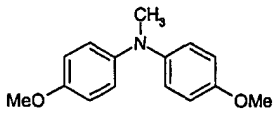
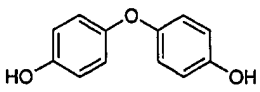
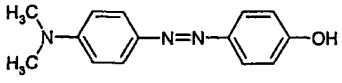
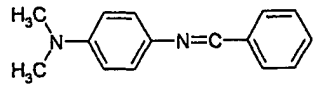
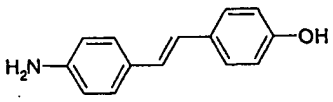
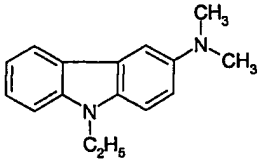
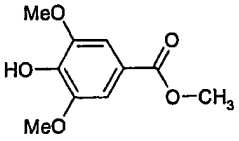
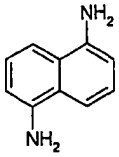
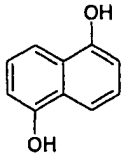
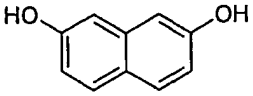
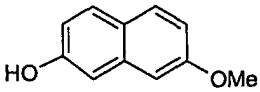
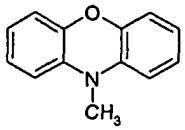
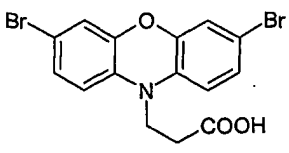
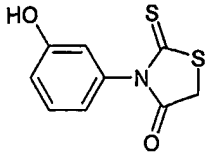
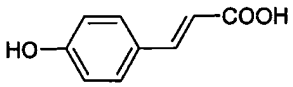
	3,7-Dibromo-phenoxazine-10-propionic acid		Novozymes	> 10^5
	3-(3-Hydroxy-phenyl)rhodanine		Sima-Aldrich	10^2-10^5

Table 6. Enhancing agents with anti-microbial activity mediated by PpL:

CAS no.	Name		Supplier	CFU reduction
123-30-8	4-aminophenol		Aldrich	> 10^5
92-88-6	4,4'-Biphenol, 97%		Aldrich	10^2-10^5
54827-17-7	3,3',5,5'-Tetra-methylbenzidine		Acros	10^2-10^5
6369-04-6	4,4'-Diamino-diphenylamine sulfate, tech. 85%		Janssen Chemica	> 10^5
27151-57-1	4,4'-Dimethoxy-N-methyldiphenyl-amine		Lancaster	10^2-10^5
1965-09-9	4,4'-Dihydroxy-diphenyl ether		Sigma-Aldrich (Acros)	> 10^5
2496-15-3	4-Hydroxy-4'-dimethylamino azobenzene		TCI	> 10^5

889-37-2	N'-Benzylidene-N,N-dimethyl-p-phenylenediamine		Sigma-Aldrich	10^2-10^5
836-44-2	4-Amino-4'-hydroxystilbene		Lancaster	10^2-10^5
	3-Dimethylamino-9-ethylcarbazole		Novozymes	> 10^5
884-35-5	Methylsyringate		Lancaster	10^2-10^5
2243-62-1	1,5-Diamino-naphthalene		Novozymes	> 10^5
83-56-7	1,5-Dihydroxy-naphthalene		Novozymes	10^2-10^5
582-17-2	2,7-Dihydroxy-naphthalene		Novozymes	10^2-10^5
5060-82-2	7-Methoxy-2-naphthol, 98%		Aldrich	10^2-10^5
25782-99-4	10-Methyl-phenoxazine		Novozymes	10^2-10^5
	3,7-Dibromo-phenoxazine-10-propionic acid		Novozymes	10^2-10^5

3-(3-Hydroxy-phenyl)rhodanine		Sima-Aldrich	10^2-10^5
p-curmaric acid		Novozymes	10^2-10^5

Reagents

50 mM HEPES buffer (Sigma Catalogue # H3375) pH 7.00

LB Bouillon was from Merck, #0285. 25 g mix was added to 1000 ml water and then autoclaved.

The laccases used are described in:

Myceliophthora thermophila (MtL)	WO 95/33836
Rhizoctonia solani (RsL)	WO 95/07988
Coprinus cinereus (CcL)	WO 97/08325
Polyporus pinsitus (PpL)	WO 96/00290

- which is hereby incorporated by reference.

Microtiter plates (96 well and 384 well plates) were obtained from Nalge Nunc International (Denmark). Enhancing agents were supplied as indicated in Table 3-6.

EXAMPLE 2

Antimicrobial activity of mediators oxidized by peroxidase

Antibacterial activity of 4-aminophenol, 4-hydroxy-4′-dimethylamino azobenzene, 4,4′-biphenol, 10-methylphenoxazine and 4,4′-dihydroxydiphenyl ether was evaluated when oxidized by *Coprinus cinereus* peroxidase (rCiP) (available from Novozymes A/S).

Antimicrobial activity was determined on *Pseudomonas putida* (ATCC 12633),

the cells were grown in Tryptone Soy Broth, TSB (Oxoid CM129) overnight, washed two times in sterile 0.9% saline and suspended in 50 mM HEPES buffer (Sigma H3375) at pH 7 to a cell concentration of approximately 10^6 CFU/ml. The mediators were added to the cell suspension to a final concentration of 50 µM, rCiP was added to the concentration 4 POXU/L and the enzyme reaction was started by addition of hydrogen peroxide to a final concentration of 0.5 mM. Antimicrobial activity was determined after 20 minutes incubation at 40°C, by a 10-fold dilution of the cell suspension into TSB substrate and incubation overnight. Antimicrobial activity was determined as $\log_{10}$ reduction, thus a $\log_{10}$ reduction of 2 corresponds to a kill of 99%.

Table 7: Antimicrobial activity of mediators oxidized by peroxidase.

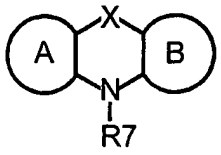
Mediator	Log_{10} reduction
4-amino phenol	2.5
4-hydroxy-4´-dimethylamino azobenzene	6*
4,4´biphenol	1.5
10-methylphenoxazine	3
4,4´-dihydroxydiphenyl ether	2.5

* corresponds to a total kill

None of the evaluated mediators showed any antimicrobial activity without rCiP or when combined with hydrogen peroxide.

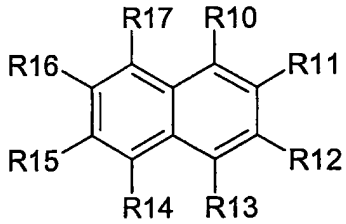
CLAIMS

1. An enzymatic antimicrobial composition comprising a phenol oxidizing enzyme system and an enhancing agent of a formula selected from the group consisting of:



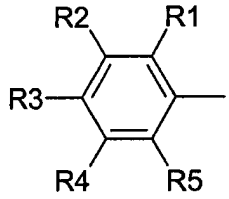
C - X - **D**;

E - R6; and



(F)

in which C, D, and E independently of each other are:



and R1, R2, R3, R4, R5, R6, R7, R10, R11, R12, R13, R14, R15, R16, R17 independently of each other are H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl; and X is a single bond or NH, NCH_3, NC_2H_5, O, S, N=N, CH=N, or CH=CH; and A and B are six membered aromatic rings, and are independently of each other be substituted with H, OH, C_{1-8}-alkyl, acyl, SO_3H, NO_2, CN, Cl, Br, F, NHR8, $N(R8)_2$, OR9, C_{1-8}-alkyl-OR9, or C_{1-8}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

2. An enzymatic antimicrobial composition according to claim 1, in which R1, R2, R3, R4, R5, R6, R7, R10, R11, R12, R13, R14, R15, R16, R17 independently of each other are H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl; and X is a single bond or NH, NCH_3, NC_2H_5, O, S, N=N, CH=N, or CH=CH; and A and B are independently

of each other be substituted with H, OH, C_{1-4}-alkyl, acyl, NO_2, Cl, Br, NHR8, $N(R8)_2$, OR9, C_{1-4}-alkyl-OR9, or C_{1-4}-alkyl-OOR9; wherein R8, and R9 are H, C_{1-4}-alkyl or acyl.

3. An enzymatic antimicrobial composition according to claim 1, in which one or more carbon atoms of the aromatic rings of A, B, C, D, E, and F are substituted with N or S, thus rendering said aromatic ring a heterocyclic ring.

4. An enzymatic antimicrobial composition according to claim 1, in which at least one of R1, R2, R3, R4, and R5 are H.

5. An enzymatic antimicrobial composition according to claim 1, in which at least one of R10, R11, R12, R13, R14, R15, R16, and R17 are H.

6. A composition according to claim 1, in which the enhancing agent is selected from the group consisting of:
4-aminophenol;
p-Coumaric acid;
4,4'-Biphenol;
3,3',5,5'-Tetramethylbenzidine;
4,4'-Diaminodiphenylamine sulfate;
4,4'-Dimethoxy-N-methyl-diphenylamine;
4,4'-Dihydroxydiphenyl ether;
4-Hydroxy-4'-dimethylamino azobenzene;
N'-Benzylidene-N,N-dimethyl-p-phenylenediamine;
4-Amino-4'-hydroxystilbene;
4'-Bromo-4-(dimethylamino)-chalcone;
3-Dimethylamino-9-ethylcarbazole;
Harmine, HCl H_2O;
Methylsyringate;
Propyl sinapate;
1,5-Diaminonaphthalene;
1,5-Dihydroxynaphthalene;

2,7-Dihydroxynaphthalene;
7-Methoxy-2-naphthol;
2-Hydroxy-1-naphthaldehyde;
2-Hydroxy-1-naphthoic acid;
8-Aminoquinoline;
10-Methylphenoxazine; and
3,7-Dibromophenoxazine-10-propionic acid.

7. A composition according to any of claims 1-6, in which the phenol oxidizing enzyme system is a peroxidase and a hydrogen peroxide source.

8. A composition according to claim 7, wherein the peroxidase is horseradish peroxidase, soybean peroxidase or a peroxidase enzyme derived from *Coprinus*, *Bacillus*, or *Myxococcus*.

9. A composition according to claim 8, wherein the peroxidase is derived from *Coprinus cinereus* or *Coprinus macrorhizus*.

10. A composition according to claim 7, wherein the hydrogen peroxide source is hydrogen peroxide or a hydrogen peroxide precursor.

11. A composition according to any of claims 1-6, in which the phenol oxidizing enzyme system is a laccase or a laccase related enzyme together with oxygen.

12. A composition according to claim 11, in which the laccase is a microbial laccase.

13. A composition according to claim 12, wherein the laccase is derived from *Coprinus*, *Myceliophthora*, *Polyporus*, *Pycnoporus*, *Scytalidium* or *Rhizoctonia*.

14. A composition according to claim 13, wherein the laccase is derived from *Coprinus cinereus*, *Myceliophthora thermophila*, *Polyporus pinsitus*, *Pycnoporus cinnabarinus*, *Scytalidium thermophilum* or *Rhizoctonia solani*.

15. The composition according to any of claims 1-6, wherein said composition is an aqueous composition.

16. The composition according to claim 15, wherein the concentration of the phenol oxidizing enzyme is in the range of from 0.001-10 mg enzyme protein per liter.

17. The composition according to claim 15, wherein the concentration of the enhancing agent corresponds to 1-1000 μM.

18. The composition according to any of claims 1-6, wherein said composition is a granulate.

19. A method for killing or inhibiting microbial cells comprising treating said microbial cells with the composition according to any of claims 1-18.

20. A detergent composition comprising a surfactant and the composition according to any of claims 1-6.

21. A method of inhibiting micro-organisms present in laundry, wherein the laundry is treated with a soaking, washing or rinsing liquor comprising the composition according to any of claims 1-6.

22. A method of preserving a cosmetic product, wherein an effective amount of the composition according to any of claims 1-6 is incorporated into the cosmetic product.

23. The method according to claim 22, wherein the cosmetic product is a mouth wash composition, a cosmetic liquid or gel or paste, an eye lotion, an antiperspirant, a deodorant, a nasal spray, an eye ointment, an ointment or cream, a foot bath salt.

24. Use of the composition according to any of claims 1-6 for cleaning or disinfection of contact lenses.

25. A method of cleaning, disinfecting or inhibiting microbial growth on a hard sur-

face, wherein the surface is contacted with the composition according to any of claims 1-6.

26. The method according to claim 25, wherein the hard surface is a process equipment such as a member of a cooling tower, a water treatment plant, a dairy, a food processing plant, a chemical or pharmaceutical process plant.

27. The method according to claim 25, wherein the hard surface is a surface of water sanitation equipment.

28. The method according to claim 25, wherein the hard surface is a surface of equipment for paper pulp processing.

29. Use of the composition according to any of claims 1-6 in a cleaning-in-place system.

30. Use of the composition according to any of claims 1-6 for disinfection of water systems.

31. The use according to claim 30 for disinfection of water systems in paper pulp processing.

32. Use of the composition according to any of claims 1-6 for preserving paint.

INTERNATIONAL SEARCH REPORT

ıtional Application No
PCT/DK 01/00315

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01N65/00 A01N63/02 A01N63/00 //(A01N65/00,43:84,43:78, 43:42,43:38,37:40,37:38,35:10,35:04,33:26,33:10,33:06,31:16, 31:08),(A01N63/02,43:84,43:78,43:42,43:38,37:40,37:38,35:10,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	page 17, line 3 - line 17	1-32

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Date of the actual completion of the international search	Date of mailing of the international search report
9 July 2001	20/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Lamers, W

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INTERNATIONAL SEARCH REPORT

International Application No
../DK 01/00315

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 35:04,33:26,33:10,33:06,31:16,31:08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	page 21, line 1 - line 9 page 22, line 6 -page 23, line 24	1-32

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 2001	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lamers, W

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International Application No
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